



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> A61K 39/35, C12N 1/21, 15/70, 15/79, 15/86, G01N 33/53	<b>A1</b>	<b>(11) International Publication Number:</b> WO 96/14089 <b>(43) International Publication Date:</b> 17 May 1996 (17.05.96)
<b>(21) International Application Number:</b> PCT/US95/13658 <b>(22) International Filing Date:</b> 10 October 1995 (10.10.95) <b>(30) Priority Data:</b> 08/333,952 3 November 1994 (03.11.94) US <b>(71) Applicant:</b> ZOOGEN, INC. [US/US]; Suite 4, 1105 Kennedy Place, Davis, CA 95616 (US). <b>(72) Inventors:</b> SVERLOW, Genadie, G.; 249 Wrentham Drive, Vacaville, CA 95688 (US). HALVERSON, Joy, L.; 2442 Elendil Lane, Davis, CA 95615 (US). <b>(74) Agents:</b> ROWLAND, Bertram, I. et al.; Flehr, Hohbach, Test, Albritton & Herbert, Suite 3400, 4 Embarcadero Center, San Francisco, CA 94111-4187 (US).		<b>(81) Designated States:</b> AU, CA, JP, NZ, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> METHODS AND COMPOSITIONS FOR THE DIAGNOSIS OF FLEA ALLERGIES  <b>(57) Abstract</b>  Methods are described for the isolation, characterization, and expression of flea allergens. cDNA clones encoding three major <i>Ctenocephalides felis felis</i> allergens are provided (pCten f I, pCten f II, pCten f III). The clones contain coding regions, and structural features involved in the transcription and translation of the flea allergens. The proteins encoded by the cDNAs have molecular weights of approximately 15,000, 16,000, and 11,000 daltons respectively. The recombinant proteins are shown to retain the antigenicity of the native molecule.		

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## METHODS AND COMPOSITIONS FOR THE DIAGNOSIS OF FLEA ALLERGIES

### INTRODUCTION

#### Technical Field

The field of this invention is the diagnosis of flea allergies.

#### Background

Allergens are a group of antigens that induce an immediate hypersensitivity reaction following interaction with cell-bound immunoglobulin E (IgE) molecules. Allergen extracts are frequently administered intradermally to detect or confirm the allergic status of a patient. Patients allergic to a particular antigen will respond with an inflammatory reaction at the site of the injection. Allergens have also been used in laboratory tests for allergy diagnosis by detecting the presence of allergen-specific IgE in the serum of patients.

Allergen extracts are frequently administered in controlled amounts and intervals (hyposensitization) as treatment for allergy. Recent evidence suggests that allergen peptides may act on T cells to uncouple the cellular interactions resulting in IgE-induced mast cell degranulation and inflammation. Allergy hyposensitization can be very effective, is easy to administer, and is well tolerated by a majority of patients.

Fleas, *Ctenocephalides felis felis* and others, are now recognized as a major cause of physiological disorders among mammals. These insects are ectoparasites that attack dogs, cats, and humans. Certain species (*i.e.*, dogs and cats), and individuals of these species are more allergic to fleabites than others, resulting in a clinical disorder called flea allergy dermatitis (FAD) or flea bite hypersensitivity. The hallmark of FAD is intense pruritis (itching) not only at the site of the flea bite but in a distinctive, body-wide distribution. This allergic reaction is a systemic response to a variety of protein substances in the oral secretions which the flea injects intradermally when it bites. Flea allergy dermatitis is the most common skin malady of the pet dog population of the United States, with as many as 8-10 per cent of pet dogs living in temperate climates being affected. In addition, greater than 50 per cent of dogs with other skin diseases, such as pyoderma and seborrhea, have FAD as a major contributing factor. FAD is a relentless annoyance to dogs, cats and their owners, causing intense pruritus. The obsessive scratching and chewing produces open sores and areas of hair loss. Chronic

FAD leads to scarring and permanent bald spots and is often associated with seborrhea, giving the dog a foul odor which pervades the household. Flea allergy also is recognized as a contributory cause of the common dermatitis of man known as papular urticaria.

5        There has been an intense interest in treatment methods for FAD. Presently, the control is primarily insecticidal. Various strong chemicals and medications are used on dogs. The large range of chemicals that are on the market include chlorinated hydrocarbons, carbamates, organophosphates, pyrethroids, and insect growth regulators. However, some dogs with extreme ectoparasite infestations may actually go  
10 into shock and die following vigorous treatment. Because some stages of the flea's life cycle persist for months in the environment, there is a need for repeated application of chemicals and for chemicals with residual action. Some chemicals with residual action may be toxic to the animal or humans. There is also the possibility of fleas developing resistance to insecticidal materials.

15        Veterinarians use systemic corticosteroid therapy to relieve FAD symptoms temporarily. However, many dogs in temperate climates require lifelong, chronic steroid treatment resulting in undesirable and even fatal consequences. In addition, many veterinary clients are increasingly sophisticated and object to the long-term use of steroids in their pets. Veterinarians practicing in temperate climates are frustrated that  
20 they can offer no therapeutic options to their patients.

A variety of antigen preparations from fleas have been used for flea allergy testing and for hyposensitizing allergic animals. Most veterinarians use commercially available whole flea extracts in which the allergenic substances naturally found in the oral secretions of the flea are extremely dilute. Investigators have attempted to modify  
25 or purify the allergens in flea oral secretions to improve efficacy. Continued progress in FAD immunotherapy requires a source of pure, concentrated allergens.

Allergens for various diagnostic and therapeutic purposes are currently extracted from natural sources. A common cause of IgE mediated reactivity in animal patients is flea allergy dermatitis. Flea extracts represent heterogeneous mixtures and probably  
30 only a few components are allergens causing FAD. It is therefore of substantial interest for diagnosis and therapy to have a pure source of the flea allergen proteins and the DNA encoding them.

#### Relevant Literature

35        A discussion of flea allergen dermatitis may be found in Dryden and Blakemore, (1989) *Companion Anim. Pract.* 19:10-17. The source of allergen is discussed in Benjamini and Feingold (1960) *Nature* 188:959-960 and Halliwell (1990), *Clinical and*

*Immunological Aspects of Allergic Skin Diseases Animals* Advances in Vet Dermatology Animals, Philadelphia, Bailliere Tindall 106-116. Clinical symptoms in dog and man are discussed in Kieffer and Kristensen (1979) *Int. J. Dermatol.* **18**:707-712 and in Doering (1976) *Vet. Clin. North Am. Small An. Pract.* **6**: 463-473.

5 Insecticidal control of fleas is described in Dryden and Rust (1994) *Veterinary Parasitology* **52**: 1-19. Various treatments of FAD are described in Schemmer and Halliwell (1987) *Seminars in Veterinary Medicine and Surgery (Small Animal)* **2**: 195-198. Vaccination with flea antigens is described in Opdebeeck and Slacek (1993) *International Journal for Parasitology* **23**: 1063-1067.

10 The use of hyposensitization as allergy therapy with heterogeneous flea allergens is described in Halliwell (1981) *J Am Anim Hosp Assoc* **17**: 249-253; Kunkle and Milcarsky (1985) *J Am Vet Med Assoc* **186**: 677-680; and with schistosome antigens in Hellman (1994) *Eur. J. Immunol.* **24**: 415-420. Flea allergens are described in Opdebeeck *et al* International application WO 93/18788. The relative IgE and IgG  
15 response to allergens is discussed in McKeon and Opdebeeck (1994) *Inter. J. Parasitol.* **24**: 259-263. Allergen characterization is reported in Tovey and Baldo (1987) *Electrophoresis* **8**: 452-463.

The molecular characterization of various allergens may be found in Breiteneder *et al* (1992) *J Allergy Clin Immunol* **90**: 909-917; Culpepper *et al* (1992) *Molecular and*  
20 *Biochemical Parasitology* **54**: 51-62; Fang *et al* (1988) *Proc. Natl. Acad. Sci.* **85**: 895-899; and Greene *et al* (1993) *Veterinary Immunology and Immunopathology* **37**: 15-23.

### SUMMARY OF THE INVENTION

Methods and compositions are provided for performing diagnostic assays for the  
25 detection of flea allergies, utilizing the isolation, characterization, and expression of flea allergens. The diagnostic methods include intradermal skin tests and immunoassays. cDNA clones encoding major *Ctenocephalides felis felis* allergens are used to produce protein allergens. The recombinant proteins retain the antigenicity of the native molecule, and are useful in diagnostic assays. The protein compositions are also useful  
30 as therapeutics, particularly for hyposensitization of allergic patients.

### DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Methods are provided for diagnostic assays and hyposensitization therapy of flea allergies, with recombinant proteins having the antigenicity of naturally produced  
35 proteins. Methods for the isolation, characterization, and expression of flea allergens are provided. The subject methods are used to make pure compositions of protein allergens from flea species, particularly *Ctenocephalides felis felis*. The allergen

proteins are expressed from DNA constructs comprising cloned DNAs, and are immunologically reactive with allergic patients. The DNA compositions also find use in identifying proteins and the DNA sequences encoding proteins having homology to the flea allergens.

5 Allergens are compounds which cause hypersensitivity reactions in mammals, generally mediated by immunoglobulin E. The allergens of interest are proteins responsible for allergic dermatitis caused by blood sucking arthropods, e.g. *Diptera*, including mosquitos (*Anopheles sp.*, *Aedes sp.*, *Culiseta sp.*, *Culex sp.*); flies (*Phlebotomus sp.*, *Culicoides sp.*) particularly black flies, deer flies and biting midges; ticks (*Dermacenter sp.*, *Ornithodoros sp.*, *Otobius sp.*); fleas, e.g. the order *Siphonaptera*, including the genera *Xenopsylla*, *Pulex* and *Ctenocephalides*. Of particular interest are the flea species *Ctenocephalides felis felis* and *Ctenocephalides canis*. Pure allergen compositions for use in diagnosis and therapy are prepared by expression from recombinant DNA.

15 Identification of DNA encoding the subject allergens utilizes a method of screening cDNA expression libraries for reactivity with allergic patient anti-serum. Messenger RNA is isolated from the arthropod species of interest by conventional methods. The mRNA source animals may be cultured on suitable media, or may be isolated from an animal host. The mRNA is reverse transcribed and cloned into a suitable expression library, conveniently using a plasmid or lambda phage vector, e.g.  $\lambda$ gt11 and variants thereof. The library is plated under conditions which allow expression of the cloned DNA.

A preparation of antiserum from an allergic animal, usually an animal suffering from allergic dermatitis, is used to screen the library. The animal(s) chosen for an antiserum sample will be allergic to the particular insect species from which the library was made. This may be verified by monitoring the reaction to insect bites. Mammalian species susceptible to allergic dermatitis include those of the family *Canidae*, e.g. dogs, wolves, coyotes, foxes and jackals; the family *Felidae*, e.g. domestic cats, lynx, bobcat, serval, ocelot, puma, leopard, lion, tiger, jaguar, and cheetah; primates, particularly humans; equines; bovines; ovines; etc. Antisera is prepared from a biological sample, usually a blood derivative, e.g. plasma or serum. Whole serum or plasma, diluted as necessary, may be used for screening. Greater specificity may be obtained by isolating the IgE fraction, usually by affinity chromatography using reagents specific for IgE, e.g. anti-IgE antisera, Staphylococcus protein G, etc.

35 Detection of IgE binding to the library may be achieved by a number of conventional methods. It is convenient to transfer phage plaques or colonies to nitrocellulose filters, followed by binding of the IgE antiserum to the filters. The

presence of bound antibody may be detected by use of a second stage antibody with specificity for IgE and having a detectable label, e.g. horseradish peroxidase, phosphatase, radioactive labels, *etc.* The plaques which specifically bind the antisera are isolated, and the cDNA insert further characterized as to DNA sequence, antisera reactivity, *etc.*

The subject methods have been used to isolate genes encoding three major allergens from *Ctenocephalides felis felis*, designated Cten f I (SEQ ID NO:1), Cten f II (SEQ ID NO:3) and Cten f III (SEQ ID NO:5), collectively referred to as Cten f genes. "Genes" shall be intended to mean the nucleotide sequences encoding the specific protein allergens, as well as adjacent 5' and 3' non-coding nucleotide sequences involved in the regulation of expression of the protein encoded by the genes, and will include up to about the length of the mature mRNA. Also included in the corresponding genomic sequence, including introns, and may include up to 1 kb of flanking genomic DNA at either the 5' or 3' end, and as much as 10 kb of flanking genomic sequence. These non-coding sequences include terminator and polyadenylation sequences, regulatory protein binding sequences, transcriptional sequences, and the like.

Cten f I (SEQ ID NO:1) contains an open reading frame from nucleotide 47 to nucleotide 392, which encodes a polypeptide of approximately 13,000 daltons (SEQ ID NO:2). Cten f II (SEQ ID NO:3) contains an open reading frame from nucleotide 58 to nucleotide 421, which encodes a polypeptide of approximately 14,000 daltons (SEQ ID NO:4). Cten f III (SEQ ID NO:5) contains an open reading frame from nucleotide 65 to nucleotide 293, which encodes a polypeptide of approximately 9,000 daltons (SEQ ID NO:6). A comparison of the DNA sequence with a database of known sequences reveals no obvious similarities between the three Cten f sequences, or with other sequences.

The nucleic acid compositions of the subject invention may be genomic or cDNA sequences encoding all or a part of the subject flea allergens. In order to obtain genomic clones, the cDNA gene may then be used to isolate the genomic gene in accordance with conventional techniques. (See, for example, Molecular Cloning: A Laboratory Manual, 2nd ed., J. Sambrook, E. F. Fritsch, T. Maniatis, CSHL, Cold Spring Harbor, NY, 1989). Fragments may be obtained of the cDNA or genomic sequence by chemically synthesizing oligonucleotides in accordance with conventional methods, by restriction enzyme digestion, by PCR amplification, *etc.* For the most part, fragments will be of at least 12 nt, more usually at least 18 nt. Preferably fragments will include a functional epitope. The sequence providing for a functional epitope can be determined by expression of the sequence, and assaying for reactivity of the expression product with allergen specific antibodies by conventional immunoassay.

The DNA sequences may be obtained in substantial purity, and will be obtained as a sequence other than a sequence of an intact chromosome. Usually, the DNA will be obtained substantially free of other nucleic acid compounds which do not include a Cten f sequence or fragment thereof, generally being at least about 50%, usually at least about 90% pure and are typically "recombinant", *i.e.* flanked by one or more nucleotides with which they are not normally associated with on a natural chromosome.

The DNA sequences may be used in a variety of ways. They may be used as probes for identifying homologous allergens from other species causing allergic dermatitis, particularly other blood-sucking arthropods, as previously described. Homologous sequences are those with substantial sequence similarity to Cten f sequences, *i.e.* at least 80%, preferably at least 90%, more preferably at least 95% sequence identity with the nucleotide sequence of a Cten f sequence. Allergens from other flea species, *i.e.* *Xenopsylla sp.*, *Pulex sp.*, *Ctenocephalides canis*, *etc.* are of particular interest. Such homologous nucleic acid sequences will be detected by hybridization under low stringency conditions, for example, at 50° C and 10XSSC (0.9 M saline/0.09 M sodium citrate) and remain bound when subject to washing at 55° C with 1XSSC.

The DNA may also be used to identify cells or organs which are expressing Cten genes. The manner in which one probes cells for the presence of particular nucleotide sequences, particularly as DNA, mRNA or cDNA, is well-established in the literature and does not require elaboration here. Conveniently, mRNA may be isolated free of DNA, and by using reverse transcriptase and PCR with primers specific for the various allergens, the cDNAs of the allergens may be expanded, separated on gel electrophoresis and then probed using Southern blotting or sequencing. Other techniques may also find use.

For expression, the DNA sequences may be inserted into an appropriate expression vector, where the native transcriptional initiation region may be employed or an exogenous transcriptional initiation region, *i.e.* a promoter other than the promoter which is associated with the gene in the normally occurring chromosome. The promoter may be introduced by recombinant methods *in vitro*, or as the result of homologous integration of the sequence into a chromosome. A wide variety of transcriptional initiation regions are known for a wide variety of expression hosts, where the expression hosts may involve prokaryotes or eukaryotes, particularly *E. coli*, *B. subtilis*, mammalian cells, such as CHO cells, COS cells, monkey kidney cells, lymphoid cells, particularly human cell lines, and the like. Generally a selectable marker operative in the expression host will be present. The promoter may be operably linked to the coding sequence of the genes of interest so as to produce a translatable mRNA



transcript encoding Cten f I, II, and III. Expression vectors have convenient restriction sites located near the promoter sequence so as to provide for the insertion of nucleic acid sequences encoding heterologous proteins. The promoters in suitable expression vectors may be either constitutive or inducible. Expression vectors for the production of fusion proteins, where the exogenous fusion peptide provides additional functionality, *i.e.* increased protein synthesis, stability, reactivity with defined antisera, an enzyme marker, *e.g.*  $\beta$ -galactosidase, *etc.*, are of particular interest.

Expression cassettes may be prepared comprising the transcription initiation region, which may be constitutive or inducible, with or without an enhancer sequence, including the endogenous or heterologous enhancer sequence, the gene encoding the subject allergens or fragment thereof, and a transcriptional termination region, optionally having a signal for attachment of a poly A sequence. The gene may be genomic, including the native introns, or cDNA gene, or portion thereof. Of particular interest is the use of sequences which allow for the expression of functional epitopes, usually at least about 24 nucleotides in length, more usually at least about 48 nucleotides in length, and up to the complete open reading frame of the gene. A functional epitope is an allergen polypeptide which is sufficient to bind allergen specific antibodies. Functional epitopes can be assayed by testing the binding of the polypeptide in a convenient immunoassay with allergen specific antibodies.

After introduction of the DNA, the cells containing the construct may be selected by means of a selectable marker, the cells expanded and then used for expression. Where secretion is desired, a signal peptide may be joined to the sequence encoding the subject proteins or fragments thereof, whereby the protein will be expressed, translocated through the cell membrane, and processed to remove the signal peptide.

The expression cassettes may be introduced into a variety of vectors, where the vectors will normally be characterized by the ability to provide selection of cells comprising the expression vectors. The vectors may provide for extrachromosomal maintenance, particularly as plasmids in bacteria or viruses in eukaryotic cells, or for integration, particularly in mammalian cells. Where extrachromosomal maintenance is desired, an origin sequence will be provided for the replication of the plasmid, which may be a low- or high-copy plasmid. A wide variety of markers are available for selection, particularly those which protect against toxins, more particularly against antibiotics. The particular marker which is chosen will be selected in accordance with the nature of the host, where in some cases, complementation may be employed with auxotrophic hosts, *e.g.* yeast. Introduction of the DNA construct may be by any convenient means, *e.g.* calcium-precipitated DNA, electroporation, fusion, transfection, infection with viral vectors, *etc.*

The subject polypeptide compositions will include the entire Cten f proteins, having the amino acid sequences Cten f I (SEQ ID NO:2), Cten f II (SEQ ID NO:4) and Cten f III (SEQ ID NO:6), collectively referred to as Cten f proteins. Also included are  
5 useful fragments thereof, usually having at least about 8 amino acids, more usually at least about 16 amino acids, particularly involving all or a major portion of an epitope. Purity will be at least about 50 wt.% of the protein present, preferably at least about 90 wt.%, more preferably substantially free of other proteins, particularly other flea or diptera proteins and cell debris. Protein compositions for injection will desirably be free  
10 of bacterial endotoxins. The allergens may be formulated as a single species, or as a cocktail comprising one or more of the Cten f proteins. A cocktail will usually comprise at least about 10% total protein weight of each of the allergen proteins.

The subject compositions may be modified in a variety of ways, such as conjugation with labels, *e.g.* radioisotopes, particles, *e.g.* magnetic, specific binding  
15 pair members, *e.g.* biotin and avidin, enzymes, fluorescers, *etc.*, particularly labels which provide, directly or indirectly, a detectable signal. The subject compositions may be conjugated to polypeptides or proteins, either fused or through a chemical linker to provide novel properties to the protein, *e.g.* extended lifetime, attachment to a target site by means of a ligand or antibody, or the like.

20 The subject compositions may be used for producing antibodies specific for one or more epitopes of the Cten f proteins. The antibodies may be produced in accordance with conventional ways, immunization of a mammalian host, *e.g.* mouse, rat, guinea pig, cat, dog, *etc.*, fusion of resulting splenocytes with a fusion partner for immortalization and screening for antibodies having the desired affinity to provide  
25 monoclonal antibodies having a particular specificity. These antibodies can be used for affinity chromatography, for identifying other allergens having similar epitopes to the subject proteins, and the like. The antibodies may find particular use as a competitor for patient anti-allergen antibodies in diagnostic assays. Those antibodies may be labeled with radioisotopes, enzymes, fluorescers, chemiluminescers, or other label which will  
30 allow for detection of complex formation between the labeled antibody and its complementary epitope.

The subject polypeptide compositions are particularly useful for diagnosis and treatment of flea allergies. Patients in which such allergies may be diagnosed include  
35 canines, felines and primates, particularly humans and domesticated cats and dogs. In particular, the Cten f allergens have been shown to react strongly with IgE from allergic canines, and are useful in diagnosing such allergies. The polypeptide compositions for

diagnosis and treatment will be produced in accordance with the previously described methods. Formulations for injection will comprise a physiologically-acceptable medium, such as saline, PBS, aqueous ethanol, aqueous ethylene glycols, or the like. In many assays a cocktail of allergens will be used, usually from a single arthropod species. However, for therapy it may be desirable to perform assays to determine the particular polypeptide species responsible for the allergic reaction, in which case a formulation will include a single polypeptide species.

Allergy diagnosis may be performed by a number of methods. The different methods all determine the presence of an IgE response to the allergen in a patient, where a positive response is indicative of an allergic condition. The IgE response may be determined by determination of antibody binding, or by the presence of a response to intradermal challenge with antigen.

In one method, a dose of allergen, formulated as a cocktail of Cten f proteins or as individual Cten f protein species, in a suitable medium is injected subcutaneously into the patient. The dose of antigen will usually be at least about 0.05  $\mu$ g of protein, and usually not more than about 5  $\mu$ g of protein. A control comprising medium alone, or an unrelated protein will be injected nearby at the same time. The site of injection is examined after a short period of time, usually at least about 15 minutes and not more than about 45 minutes, for the presence of a wheal. The wheal at the site of allergen injection is compared to that at the site of the control injection, usually by measuring the size of the wheal. The skin test readings may be assessed by a variety of objective grading systems. A positive result for the presence of an allergic condition will show an increased diameter at the site of allergen injection as compared to the control, usually at least about 50% increase in size, more usually at least 100% increase in size. It is standard procedure to carry out tests in duplicate or triplicate.

An alternative method for diagnosis depends on the *in vitro* detection of binding between IgE in a patient sample and the subject allergens, either as a cocktail of Cten f proteins or as individual Cten f protein species, where the presence of specific binding is indicative of an allergic condition. Measuring the concentration of an allergen specific IgE in a sample or fraction thereof may be accomplished by a variety of specific assays. In general, the assay will measure the reactivity between a patient sample, usually blood derived, generally in the form of plasma or serum. To differentiate between allergen specific IgG and IgE antibodies, the IgE fraction may be purified from the patient sample by various affinity methods, e.g. Staph protein G, anti-IgE antibodies, etc., or more conveniently, a specific anti-IgE reagent may be used for detection of binding. The patient sample may be used directly, or diluted as appropriate, usually about 1:10

and usually not more than about 1:10,000. Immunoassays may be performed in any physiological buffer, *e.g.* PBS, normal saline, HBSS, dPBS, *etc.*

In a preferred embodiment, a conventional sandwich type assay is used. A sandwich assay is performed by first attaching the allergen to an insoluble surface or support. The allergen may be bound to the surface by any convenient means, depending upon the nature of the surface, either directly or through specific antibodies. The particular manner of binding is not crucial so long as it is compatible with the reagents and overall methods of the invention. They may be bound to the plates covalently or non-covalently, preferably non-covalently.

The insoluble supports may be any compositions to which allergen polypeptides can be bound, which is readily separated from soluble material, and which is otherwise compatible with the overall method of measuring IgE. The surface of such supports may be solid or porous and of any convenient shape. Examples of suitable insoluble supports to which the receptor is bound include beads, *e.g.* magnetic beads, membranes and microtiter plates. These are typically made of glass, plastic (*e.g.* polystyrene), polysaccharides, nylon or nitrocellulose. Microtiter plates are especially convenient because a large number of assays can be carried out simultaneously, using small amounts of reagents and samples.

Before adding patient samples or fractions thereof, the non-specific binding sites on the insoluble support *i.e.* those not occupied by allergen, are generally blocked. Preferred blocking agents include non-interfering proteins such as bovine serum albumin, casein, gelatin, and the like. Alternatively, several detergents at non-interfering concentrations, such as Tween, NP40, TX100, and the like may be used.

Samples, fractions or aliquots thereof are then added to separately assayable supports (for example, separate wells of a microtiter plate) containing support-bound allergen. Preferably, a series of standards, containing known concentrations of IgE is assayed in parallel with the samples or aliquots thereof to serve as controls.

Generally from about 0.001 to 1 ml of sample, diluted or otherwise, is sufficient, usually about 0.01 ml sufficing. Preferably, each sample and standard will be added to multiple wells so that mean values can be obtained for each. The incubation time should be sufficient for IgE molecules to bind the insoluble allergen. Generally, from about 0.1 to 3 hr is sufficient, usually 1 hr sufficing.

After incubation, the insoluble support is generally washed of non-bound components. Generally, a dilute non-ionic detergent medium at an appropriate pH, generally 7-8, is used as a wash medium. From one to six washes may be employed, with sufficient volume to thoroughly wash non-specifically bound proteins present in the sample.

After washing, a solution containing patient antibody specific second receptor, in most cases patient IgE specific second receptor, is applied. The receptor may be any compound which binds patient antibodies with sufficient specificity such that it can be distinguished from other components present. In a preferred embodiment, second  
5 receptors are antibodies specific for patient IgE, either monoclonal or polyclonal sera, e.g. mouse anti-human IgE, mouse anti-dog IgE, rabbit anti-cat IgE, etc.

IgE specific receptors may be labelled to facilitate direct, or indirect quantification of binding. Examples of labels which permit direct measurement of second receptor binding include radiolabels, such as  $^3\text{H}$  or  $^{125}\text{I}$ , fluorescers, dyes,  
10 beads, chemiluminescers, colloidal particles, and the like. Examples of labels which permit indirect measurement of binding include enzymes where the substrate may provide for a colored or fluorescent product. In a preferred embodiment, the second receptors are antibodies labeled with a covalently bound enzyme capable of providing a detectable product signal after addition of suitable substrate. Examples of suitable  
15 enzymes for use in conjugates include horseradish peroxidase, alkaline phosphatase, malate dehydrogenase and the like. Where not commercially available, such antibody-enzyme conjugates are readily produced by techniques known to those skilled in the art.

Alternatively, the second receptor may be unlabeled. In this case, a labeled second receptor-specific compound is employed which binds to the bound second  
20 receptor. Such a second receptor-specific compound can be labelled in any of the above manners. It is possible to select such compounds such that multiple compounds bind each molecule of bound second receptor. Examples of second receptor/second receptor-specific molecule pairs include antibody/anti-antibody and avidin (or streptavidin)/biotin. Since the resultant signal is thus amplified, this technique may be advantageous where  
25 only a small amount of IgE is present. An example is the use of a labeled antibody specific to the second receptor. More specifically, where the second receptor is a rabbit anti-allotypic antibody, an antibody directed against the constant region of rabbit antibodies provides a suitable second receptor specific molecule. The anti-immunoglobulin will usually come from any source other than human, such as ovine,  
30 rodentia, particularly mouse, or bovine.

The volume, composition and concentration of IgE specific receptor solution provides for measurable binding to the IgE already bound to receptor. Generally, the same volume as that of the sample is used: from about 0.001 to 1 ml is sufficient, usually about 0.1 ml sufficing. The concentration will generally be sufficient to saturate  
35 all IgE potentially bound to allergen. When antibody ligands are used, the concentration generally will be about 0.1 to 50  $\mu\text{g/ml}$ , preferably about 1  $\mu\text{g/ml}$ . The solution containing the second receptor is generally buffered in the range of about pH 6.5-9.5.

The solution may also contain an innocuous protein as previously described. The incubation time should be sufficient for the labeled ligand to bind available molecules. Generally, from about 0.1 to 3 hr is sufficient, usually 1 hr sufficing.

After the second receptor or second receptor-conjugate has bound, the insoluble support is generally again washed free of non-specifically bound second receptor, essentially as described for prior washes. After non-specifically bound material has been cleared, the signal produced by the bound conjugate is detected by conventional means. Where an enzyme conjugate is used, an appropriate enzyme substrate is provided so a detectable product is formed. More specifically, where a peroxidase is the selected enzyme conjugate, a preferred substrate combination is  $H_2O_2$  and is O-phenylenediamine which yields a colored product under appropriate reaction conditions. Appropriate substrates for other enzyme conjugates such as those disclosed above are known to those skilled in the art. Suitable reaction conditions as well as means for detecting the various useful conjugates or their products are also known to those skilled in the art. For the product of the substrate O-phenylenediamine for example, light absorbance at 490-495 nm is conveniently measured with a spectrophotometer.

Generally the amount of bound IgE detected will be compared to control samples from non-allergic animals. The presence of increased levels of allergen specific IgE is indicative of an allergic condition, usually at least about a 10 fold increase will be taken as a positive reaction.

In some cases, a competitive assay will be used. In addition to the patient sample, a competitor to the IgE is added to the reaction mix. The competitor and the IgE compete for binding to the allergen. Usually, the competitor molecule will be labeled and detected as previously described, where the amount of competitor binding will be proportional to the amount of IgE present. The concentration of competitor molecule will be from about 10 times the maximum anticipated IgE concentration to about equal concentration in order to make the most sensitive and linear range of detection.

An alternative protocol is to provide anti-patient IgE bound to the insoluble surface. After adding the sample and washing away non-specifically bound proteins, one or a combination of the subject allergens are added, where the allergens are labeled so as not to interfere with the allergen binding to the IgE. Conveniently, fused proteins may be employed, where the allergen peptide sequence is fused to an enzyme sequence, e.g.  $\beta$ -galactosidase.

It is particularly convenient in a clinical setting to perform the immunoassay in a self-contained apparatus. A number of such methods are known in the art. The apparatus will generally employ a continuous flow-path of a suitable filter or membrane, having at least three regions, a fluid transport region, a sample region, and a measuring

region. The sample region is prevented from fluid transfer contact with the other portions of the flow path prior to receiving the sample. After the sample region receives the sample, it is brought into fluid transfer relationship with the other regions, and the fluid transfer region contacted with fluid to permit a reagent solution to pass through the sample region and into the measuring region. The measuring region may have bound to it the allergen, with a conjugate of an enzyme with an IgE specific antibody employed as a reagent, generally added to the sample before application. Alternatively, the allergen may be conjugated to an enzyme, with IgE specific antibody bound to the measurement region.

Other immunoassays are known in the art and may find use as diagnostics. Ouchterlony plates provide a simple determination of antibody binding, although they will not easily differentiate between IgE and IgG binding. Western blots may be performed on protein gels or protein spots on filters, using a detection system specific for IgE as desired, conveniently using a labeling method as described for the sandwich assay.

The subject protein composition may find application in therapy, particularly for hyposensitization of patients susceptible to flea specific allergies. These include intradermal, subcutaneous or intramuscular injections with aqueous allergen formulated in a physiologically acceptable medium, either singly or as a cocktail, or with allergen in an adjuvant, generally an alum precipitate. Adjuvants will not usually be necessary, but may be included. The use of adjuvants generally increases the interval between injections. The amount of allergen to be injected may be empirically derived, and will depend on the size of the animal, usually at least about 100 ng allergen/kilogram of body weight, and not more than about 1 mg allergen/kilogram body weight. Frequently the dose will be increased through the course of injections by as much as about ten to one hundred fold.

The number of injections in an initial series will usually be at least about 5 injections, and may be as many as about 20 injections, or until there are positive signs of improvement. The frequency of injections for an initial series will be from about every 3 to about every 7 days for an aqueous formulation and from about 7 to from about 21 days for an adjuvant formulation. After the initial series, a series of booster injections may be administered on the recurrence of symptoms. Booster injections may be administered less frequently, from about every week to as infrequently as every one to three months. It will be appreciated that therapy must be tailored to the individual's response. The total number of injections may vary according to the time of year therapy is started, and the patient's response to therapy. Booster injections after the initial series will be performed seasonally if appropriate, or with the incidence of signs of dermatitis.

There is often an advantage to giving periodic boosters during the non-allergy season for patients with a seasonal allergic pattern.

The injections are continued until either a therapeutic effect becomes evident, evidenced as a decrease in allergic symptoms, e.g. pruritis, dermatitis, inflammation, etc., or blocking antibodies develop, usually detected as an increase in specific IgG titer. The detection of blocking antibodies may be performed essentially as described for the detection of IgE antibodies, using IgG specific reagents for detection.

The following examples are offered by way of illustration and not by way of limitation.

10

### EXPERIMENTAL

#### Example 1: *Ctenocephalides felis felis* cDNA Library Preparation

Messenger RNA (mRNA) was isolated encoding the Cten f I, Cten f II, and Cten f III allergens from fleas, *Ctenocephalides felis felis*. Polyadenylated mRNA was isolated, extracted, sedimented, and affinity purified on an oligo (dT)-cellulose column according to protocols of a Fast Track™ kit from Invitrogen, using the reagents and buffers provided by Invitrogen. The mRNA was reverse transcribed into cDNA, which was cloned into lambda gt11, and the resulting cDNA library was screened by using antibodies from patients with flea allergy dermatitis. Phage-producing plaques reactive with the antiserum were rescreened to finally obtain a purified clone for further examination. The protocol for mRNA isolation is as follows:

15

One gram of live fleas (~3000 fleas) in a plastic vial was partially submerged in a dry ice/ethanol bath until frozen. Fleas were ground to a powder in a prechilled mortar and pestle, keeping the fleas always in a small volume of liquid nitrogen.

Ground fleas were transferred to a 50 ml conical tube containing 15 ml of Stock Buffer and 300 microliters (μl) RNase-Protein degrader. The contents were homogenized at full speed with a Brinkman hand held homogenizer for about 30 seconds. The tube was then placed in a 45° C water bath for 1 hour.

25

Insoluble material was sedimented at 4000g for 5 minutes at room temperature, and the supernatant was transferred to a fresh tube. NaCl concentration was adjusted by adding 0.95 ml of 5M NaCl. DNA was sheared by passing lysate 3 or 4 times through a sterile plastic syringe fitted with a 21 gauge needle.

30

One oligo dT cellulose tablet was added to the tube and allowed to swell for 2 minutes before crushing with a sterile pipet. The solution was mixed well and placed on a shaker at low speed for 60 minutes. The oligo dT cellulose was pelleted at 4000g for 5 minutes at 18° C. The supernatant was removed and the pellet resuspended in 20 ml of Binding Buffer. The solution was pelleted again at 4000g for 5 minutes, the supernatant was removed and the pellet resuspended in 10 ml of Binding Buffer. The

35



solution was pelleted again and resuspended in 10 ml Low Salt Wash Buffer. The Low Salt wash was repeated two times, and after the last wash, the pellet was resuspended in 0.8 ml Low Salt Wash Buffer.

5 The sample was then pipetted into a spin column in a microfuge tube and sedimented at 18° C for 10 seconds at full speed. The sample in the spin column was resuspended in 0.8 ml Low Salt Wash Buffer. This process was repeated two additional times.

10 The spin column was placed into a new microfuge tube and 0.2 ml of Elution Buffer was added and mixed well. The tube was sedimented for 10 seconds and 0.2 ml Elution Buffer was added to the spin column and mixed well. The tube was sedimented again for 1 minute to collect all the liquid in the microfuge tube. The mRNA was precipitated by adding 0.15 volumes of 2M Sodium Acetate and 2.5 volumes of 95% ethanol. The tube was mixed well and frozen at -20° C for approximately 16 hours.

15 The tube was sedimented at full speed at 4° C in a microfuge for 15 minutes, and the supernatant was removed. To the tube, 0.5 ml of 70% ethanol was added and mixed. The tube was sedimented at full speed at 4° C for 5 minutes, and the supernatant removed. The pellet was resuspended in 20 microliters Elution Buffer. A 1% agarose gel with 1X TAE running buffer, 3 µl of the mRNA was run to determine the quality and concentration.

20 The Promega Riboclone™ kit with AMV Reverse Transcriptase was utilized to synthesize the blunt ended cDNAs. The protocol (all elements were included in the kit except mRNA) is as follows:

The following components were mixed and heated to 70° C for 5 minutes:

25 17 µl mRNA  
9 µl H<sub>2</sub>O  
5 µl Oligo dT primer

30 The mixture was cooled to 18° C for 2 minutes. 10 µl First Strand Buffer and 1µl RNasin Inhibitor (10 units/µl) was added and heated to 42° C for 2 minutes. 5 µl of 40 mM Na Pyrophosphate and 3 µl AMV Reverse Transcriptase (25 units/µl) were added at 18° C. The mixture was mixed well, sedimented quickly, and placed at 42° C for 1 hour.

35 167.5 µl Nuclease free water, 25 µl Second Strand Buffer, 6 µl E.coli DNA Polymerase (10 units/µl) and 1.5 µl E.coli RNase H (40 units/µl) were added. The mixture was mixed well, sedimented quickly, and placed at 15° C for 2 hours. It was placed at 18° C for 1 hour and then heated to 70° C for 10 minutes.

The mixture was quickly sedimented and 1 µl of T4 DNA Polymerase (10 units/µl), was added, mixed, and quickly sedimented. It was then placed at 37° C for 10 minutes.

(Note: The following steps were not performed using a kit). 10 µl 0.5 M EDTA was added, and the mixture placed on ice. 260 µl Phenol/Chloroform (1:1) was added and the phases were mixed for 1 minute. The mixture was sedimented at full speed for 3 minutes and the supernatant removed to a new tube. 260 µl 4 M ammonium acetate and 1 ml cold 95% ethanol was added. The sample was frozen on dry ice, thawed, and sedimented for 15 minutes at 4° C. The ethanol was removed and 500 µl 70% ethanol was added. The mixture was rocked 2 times to mix and then sedimented for 5 minutes at 4° C and the ethanol removed.

The pellet was resuspended in 15 µl sterile water and the following was added:

2 µl NotI/EcoRI Open Reading Frame Adapters (Invitrogen, 1mg/ml)  
2 µl 10X Ligase buffer  
1 µl T4 DNA ligase (NEB, 100 units/µl)

The solution was mixed well and quickly sedimented. The mixture was incubated at 15° C for 16 hours.

5 µl of 50 µg/ml RNase A was added. The mixture was incubated at 37° C for 10 minutes. 1 µl 0.5 M EDTA, 7.5 µl 2 M sodium acetate, and 16.5 µl sterile water were added. In addition to the above ingredients, 50 µl phenol/chloroform (1:1) were added and the phases mixed for approximately 1 minute and sedimented for 5 minutes. The top phase was transferred to a new and sterile microfuge tube. 50 µl isopropanol were added and mixed well. The solution was allowed to stand at 18° C for 10 minutes. The mixture was sedimented for 15 minutes at 4° C and the isopropanol was removed. The pellet was washed with 70% ethanol and sedimented for 5 minutes. The ethanol was removed and the pellet was resuspended in 16 µl sterile water.

2 µl of 10X Kinase Buffer (Pharmacia One-Phor-All™ plus 10mM ATP) and 1 µl T4 Polynucleotide kinase (10 units/µl) was added. The sample was mixed and placed at 37° C for 30 minutes. An additional 1 µl Kinase was added and the mixture was incubated for another 30 minutes at 37° C. 5 µl 0.5 M EDTA was added and the sample was heated to 70° C for 5 minutes. 5 µl loading dye was added and the sample was loaded on a 1X TAE, 1% Low Melting Point Agarose minigel. The sample ran about 5 cm into the gel which was stained with ethidium bromide. The gel was photographed and the cDNA greater than 500 base pairs in size was excised.

The gel slice was placed in a 2 ml reaction tube and heated to 70° C for 10 minutes, to allow the gel slice to melt. The melted mixture was incubated at 40° C for 5 minutes, and then 1/10 volume of 10X beta-agarase buffer (NEB) and 10 µl beta-agarase (NEB, 1 unit/µl) was added. The mixture was incubated at 40° C for 1 hour. The mixture was placed on ice for 5 minutes to confirm complete digestion of the agarose. An equal volume of phenol/chloroform (1:1) was added and mixed for 1

minute. The sample was mixed for 3 minutes and the top phase was removed into a new microfuge tube. 0.15 volumes of 2 M sodium acetate and an equal volume of isopropanol was added. The sample was mixed by inverting several times and placed at 4° C for 16 hours.

5 The sample was sedimented for 15 minutes at 4° C in a microfuge, and the supernatant was removed. The pellet was washed with 500 µl 70% ethanol and sedimented for 5 minutes. The supernatant was removed and the pellet was resuspended in 25ul water.

10 Ligations to phosphatased Promega λgt11/EcoRI were set up as follows (all numbers refer to microliter amounts):

#	Water	10XNEB buffer	Vector	cDNA	NEB T4 Ligase (100 units/ul)
1	2.5	0.5	1.0	0.5	0.5
2	1.5	0.5	1.0	1.5	0.5
3	-	0.5	1.0	3.0	0.5
4	3.0	0.5	1.0	-	0.5

The ligations were incubated for 3 hours at 18° C.

15 The ligations were packaged by using Gigapack II Plus packaging extracts according to manufacturer's instructions (Stratagene) and incubated for 2 hours at 18° C. 500 µl SM buffer and 30 µl chloroform was added and mixed well.

20 Y1090 cells were transfected according to the following protocol: 10 ml Luria-Bertani medium with 10mM MgSO<sub>4</sub>, 0.2% Maltose was inoculated with a single Y1090 colony. The solution was incubated with shaking at 37° C for 4-6 hours. LB + MgSO<sub>4</sub> plates were warmed to 37° C. The cells were diluted to OD<sub>600</sub> of 0.5 in 10 mM MgSO<sub>4</sub> and placed on ice. Each packaged ligation was diluted 1:10 and 1:100 in SM and placed on ice. 200 µl Y1090 cells were placed into each Falcon 2059 tubes and 1 µl of each dilution and 1 µl undiluted packaging reactions was added. The mixture was incubated at 37° C for 15 minutes and 3 ml Top Agarose (IPTG/Xgal) was added, mixed by  
25 inverting and poured onto plates. The plates were incubated at 37° C for 16 hours.

Plaques were counted to calculate the library titer. Nine clear plaques and 1 blue plaque were picked for PCR analysis. A plaque was placed in a PCR tube and 25 µl 2X PCR buffer was added. The samples were heated to 100° C in a PCR apparatus for 10 minutes. 25 µl of the following cocktail was added: lambda gt11 forward and reverse

primers (50 ng each), dNTP (250 mM final), Taq polymerase (1 unit), and water to 50  $\mu$ l, mixed well and amplified for 30 cycles of 1 minute at 94° C, 1 minute at 55° C, and 1 minute at 72° C, with a final extension of 10 minutes at 72° C. Ten  $\mu$ l of PCR product was run on a 1% agarose gel to determine the average insert size.

- 5        The library was amplified by plating 50,000 pfu/plate on each of 20 LB plates (+10mM MgSO<sub>4</sub>). The plates were incubated at 37° C for 16 hours and overlaid with 10 ml SM per plate and rocked for 4 hours at 18° C. The SM was collected from each plate and sedimented for 10 minutes at 5000 rpm to pellet the bacteria. The supernatant was stored at 4° C in 50 ml conical tubes with 2 ml chloroform/tube and in frozen
- 10       aliquots at -80° C in 7% DMSO for long term storage.

### Example 2: Sera Characterization

Serum samples were collected from over 20 dogs where flea allergy dermatitis was diagnosed visually by a veterinarian or from their clinical history of fleas and/or pruritis and chronic lumbo-sacral skin lesions. Each serum obtained was screened for high IgE titer against total flea extracts. The sera which generated the highest intensity signal to specific allergens on Western blots was further purified using either Protein G sepharose (Pharmacia) and/or preabsorption with non-recombinant *E. coli* lysate. Dog IgE was not bound to Protein G-Sepharose, while 100% of IgG, 95% of IgA, and 44% of IgM were bound to Protein G-Sepharose. Whole frozen fleas were ground with mortar and pestle. The slurry was centrifuged and the supernatant mixture was filtered through a 0.22 micron Millipore filter, aliquoted and frozen at -20° C. Approximately 1-2 micrograms of flea extract was run on a gradient SDS-PAGE (BioRad) at 35 mA for 3 hours. The proteins were electrophoretically transferred at room temperature to Hybond for approximately 2 hours at 250 mA. After the transfer, the membrane was blocked in Western buffer at 18° C for 2-3 hours. The membrane was then incubated at 18° C with the sera diluted 1:500. After 15 minute washes with Western buffer for 1 hour, the membrane was incubated with mouse monoclonal anti-canine IgE (Dr. Douglas J. DeBoer, University of Wisconsin) at 1:500 for 3 hours at 18° C. After washing the membrane as before, the final incubation was done with polyclonal anti-mouse IgG-HRP (Boehringer Mannheim) at 1:4,000. The membrane was washed as previously. The image was then developed using the enhanced chemiluminescent (ECL) Western blotting detection system (Amersham). Major allergens were identified in terms of both the frequency of the response by allergic patients and the intensity of the total IgE signal directed against these allergens.

### Example 3: Isolation of the Flea "Cten f I, II, and III" Genes

The library produced by the method of Example 1 was screened by immunoassay, using a canine anti-Cten f I, II and III antiserum. Phage were plated at approximately 15,000 plaque-forming units per 150 mm Petri plate. For immunodetection of expressed proteins in *E. coli* Y1090 transfected with lambda gt11 constructs, plaque-forming units were incubated for 3.5 hours at 42° C, then transferred onto nitrocellulose containing 10 mM isopropyl- $\beta$ -D-thiogalactopyranoside. After an incubation period of 3.5 hours at 37° C, the filters were placed in Western buffer for 3 hours at 18° C. The membrane was then incubated with the sera (RM8) diluted 1:500 at 18° C. After 15 minute washes with Western buffer for 1 hour, the membrane was incubated with mouse anti-canine IgE at 1:500 for 3 hours at 18° C. After washing the membrane as before, the final incubation was done with IgG-HRP labeled antibody at

1:4,000. The membrane was washed as previously. The membrane was then developed using the enhanced chemiluminescent (ECL) Western blotting detection system. Several phage clones reacted with the dog antiserum. Positive signals of varying intensity were seen.

5

#### Example 4: DNA Sequencing and Computer Analysis

The DNA inserts were subcloned into pCRII (Invitrogen) and DNA sequencing was performed by the chain termination method both manually and using an autosequencer (Applied Biosystems). The nucleotide sequence analysis of the cDNA insert (pCten f I, II, and III) were performed on both strands. The cDNA sequence and the deduced amino acid sequence derived from cDNA inserts are provided as follows:

Cten f I cDNA sequence	SEQ ID NO:1
Cten f I protein sequence	SEQ ID NO:2
Cten f II cDNA sequence	SEQ ID NO:3
Cten f II protein sequence	SEQ ID NO:4
Cten f III cDNA sequence	SEQ ID NO:5
Cten f III protein sequence	SEQ ID NO:6.

#### Example 5: Protein Expression

To examine the protein produced by lambda-gt11 phage, a 471bp (SEQ ID NO:1) fragment was subcloned into Invitrogen's expression vector, pRSET at the EcoRI site. An 870bp (SEQ ID NO:3) fragment was subcloned at the XhoI/KpnI site. A 620 bp (SEQ ID NO:5) fragment was subcloned into the EcoRI site.

The following media were prepared:

25

#### SOB (For 1 liter)

To 950ml of deionized water add:

20.0g	Tryptone
5.0g	Yeast Extract
0.5g	NaCl
186 mg	KCl

The solution was mixed and adjusted to pH7.0 with NaOH. The volume was adjusted to 1000 ml and sterilized by autoclaving. After autoclaving, 10 ml of sterile 1M MgCl<sub>2</sub> was added. If solid media was required, 15 g of Bacto-agar was added after adjusting the pH. Finally, ampicillin was added to a final concentration of 50 mg/ml.

JM 109 Minimal Medium (For 1 liter)

The following reagents were added to 790 ml sterile (autoclaved) deionized water:

	200.0 ml	5X M9 salts (sterile)
	10.0 ml	2M glucose (sterile)
5	0.1 ml	0.5% thiamine (sterile)
	1.0 ml	1M MgSO <sub>4</sub>
	0.1 ml	1M CaCl <sub>2</sub>

5X M9 salts (For 1 liter)

	64.0g	Na <sub>2</sub> HPO <sub>4</sub> ·7H <sub>2</sub> O
10	15.0g	KH <sub>2</sub> PO <sub>4</sub>
	2.5g	NaCl
	5.0g	NH <sub>4</sub> Cl

Sterilize by autoclaving.

SOC (For 1 liter)

- 15 Identical to SOB with the exception of allowing to cool to ~60° C and adding 10 ml of 50% glucose after autoclaving.

Lysis Buffer

	100 mM	Tris.Cl (pH 7.8)
	150 mM	NaCl
20	5 mM	MgCl <sub>2</sub>
	1.5%	bovine serum albumin
	1 mg/ml	pancreatic DNAase I
	40 mg/ml	lysozyme

TNT Buffer

25	10 mM	Tris.Cl (pH 8.0)
	150 mM	NaCl
	0.05%	Tween 20

Western Buffer

	0.25%	Gelatin
30	1X	PBS
	0.05%	Tween 20

JM109 Competent Cells

- 200 ml SOB plus ampicillin was inoculated with a single colony of *E. coli*
- 35 JM109 (on M9 minimal media plus thiamine plate). The solution was incubated at 37° C for ~16 hours. The cells were sedimented at 3500 rpm (~4000xg) in a low speed centrifuge for 15 minutes at 0° C, using four 50 ml centrifuge bottles. The supernatant was decanted and the cells were placed on ice. 10 ml of cold, sterile water was added per bottle and placed on ice. The cells were resuspended and then placed on ice again.
- 40 The wash/sedimentation cycle was repeated for an additional four times.

Ten ml of cold, sterile 10% glycerol was added per bottle and the cells were resuspended. The wash/sedimentation was repeated for an additional two more times. The cells were resuspended in 500 ml/bottle of sterile, cold 10% glycerol and divided into 80 µl aliquots.

The ligation mixture was ethanol precipitated and resuspended in 3 ml TE buffer. Eighty ml of cold cells were added to the 3 ml of precipitated and resuspended ligation mixture. The cells were electroporated according to the Invitrogen bacterial electroporation protocol. Immediately 950  $\mu$ l SOC medium was added and mixed well. The mixture was transferred to a sterile 15 ml conical tube and incubated at 37° C, shaking for 1 hour. 200  $\mu$ l of the mixture was plated on SOB plus ampicillin plates and incubated at 37° C for approximately 16 hours.

500  $\mu$ l of 1 mM IPTG was spread on surface-dried SOB plus ampicillin plates to create IPTG plates. Circular nitrocellulose filters (82-mm) were immersed in 1 mM IPTG and dried completely on Whatman paper. Each IPTG filter was placed on IPTG plates.

Transformed (electroporated) colonies were replica plated onto the IPTG filters. The plates were incubated at 37° C for about 5 hours. The filters were removed and placed on damp paper towels. The colonies on the filters were exposed to chloroform vapor for 30 minutes. The filters were transferred to lysis buffer at 18° C for about 16 hours with gentle agitation.

The filters were then placed in TNT buffer for about 30 minutes at 18° C. The TNT step was repeated. Filters were transferred to fresh TNT buffer for an additional 2 hours at 18° C.

Each filter was probed with clarified serum RM8 at 1:100 dilution, overnight at 18° C. After the incubation, the filters were washed with TNT four times at 15 minutes each. The filters were then probed with mouse monoclonal anti-canine IgE at 1:500 dilution for about 3 hours at 18° C. After the filters were washed with TNT as before, they were probed with IgG-HRP anti-mouse Ig at 1:4,000 dilution for about 3 hours at 18° C.

The results were then developed as images on X-ray film with the enhanced chemiluminescence assay system (Amersham). A number of colonies reacted with the anti-sera, and were chosen for further characterization.

#### Example 6: Protein Purification and Identification

Recombinant clones expressing Cten f I, II, and III fragments in the correct orientation were detected by colony immunoassay and chosen for further analysis. Overnight cultures of transformed *E. coli* were diluted with fresh medium and grown to an OD<sub>600</sub> of 0.3 at 37° C before isopropyl- $\beta$ -D-thiogalactopyranoside was added to a final concentration of 1 mM. The cultures were incubated at 37° C for an additional 1 hour. M13/T7 helper phage was added to each culture at a concentration of 5 pfu/cell



after one hour of incubation. The complete mixtures were then incubated for 16 hours at 37° C.

After the cells were harvested from a 15 ml culture by sedimentation, the Invitrogen Xpress System Protein Purification Kit™ was used in the following steps.

5 The cells were resuspended in 3 ml of Guanidinium Lysis Buffer, pH7.8 (equilibrated to 37° C). The cell mixture was gently rocked for 30 minutes at 18° C. The cell mixture was passed through a small gauge needle to lyse the cells and shear DNA and RNA. The insoluble debris was removed by sedimentation.

10 The clarified lysate was batch bound by resuspending the pre-equilibrated column with the 3ml lysate. The column with the lysate was gently rocked for 30-60 minutes at 18° C. The resin was allowed to settle by gravity and the supernatant was discarded.

The Invitrogen protocol for column washing and elution under denaturing conditions was followed. The eluent was concentrated by using an Amicon microcon-10 unit (10,000 MW cut off ).

The concentrate was washed four times with 0.5 M Tris-HCl, pH 6.8, using the same microcon-10 unit, to remove traces of urea. The solution was concentrated to about 300 µl using the microcon-10 unit. Ten µl of purified protein was loaded on a precast 10-20% (w/v) polyacrylamide gradient gel (BioRad).

20 The proteins were transferred electrophoretically onto a nitrocellulose filter. The protein blot was blocked in Western buffer for about 3 hours at 18° C. The blot was then incubated in the patient anti-sera described in Example 2 at 1:250 dilution at 4° C for 16 hours. The membrane was washed thoroughly in Western buffer and incubated with an anti-canine IgE monoclonal at 1:500 dilution for about 3 hours at 18° C. The membrane was washed thoroughly again and probed with polyclonal anti-mouse IgG-HRP at 1:6,000 dilution for 2 hours at 18° C. The protein image was developed by using the enhanced chemiluminescence assay system (Amersham). The results showed that each of the three cDNA clones expressed a single immunoreactive protein. In all three cases the protein was the appropriate molecular weight for the fusion product.

30

It is evident from the above results that the subject invention provides for a pure and convenient source of flea allergen proteins. DNA sequences encoding major flea allergens are isolated and sequenced, and used to produce recombinant proteins. The proteins are reactive with antibodies from patients suffering from flea allergies, and so are useful in diagnosing flea allergic dermatitis, and other related allergic conditions.

35

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

5 Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

5 (i) APPLICANT: Sverlow, Genadie G.  
Halverson, Joy L.

10 (ii) TITLE OF INVENTION: Identification and Isolation of Flea  
Allergens

(iii) NUMBER OF SEQUENCES: 6

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20 (E) COUNTRY: USA  
(F) ZIP: 94111-4187

(v) COMPUTER READABLE FORM:  
(A) MEDIUM TYPE: Floppy disk  
25 (B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:  
30 (A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:  
35 (A) NAME: Rowland, Bertram I.  
(B) REGISTRATION NUMBER: 20,015  
(C) REFERENCE/DOCKET NUMBER: A-60359/BIR; ZOOG-2

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(C) TELEX: 910 277299

(2) INFORMATION FOR SEQ ID NO:1:

45 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 471 base pairs  
(B) TYPE: nucleic acid  
50 (C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

55 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:  
GAATTCTGCG GCCGCCCGTT TCTTTCTCAT TCGGAATAAA GTCGAAATGA CCTGCAAACG 60  
TCGTAATGGT GGACGTGCCA AGCACGGTAG AGGACATGTG ACCCCTGTAA GATGCACAAA 120

60

TTGTGCTCGA TGTGTCCCCA AAGACAAGGC TATCAAGAAG TTCGTTATTA GGAACATCGG 180  
 CGAAGGAGCT GCTGTACGTG ATATCACCGA GGCTTCTGTT TATTCCGCTT ACGTCTTACC 240  
 5 TAAGTTGTAT GCTAAGTTAC ACTACTGCGT TTCCTGCGCT ATTCACTCTA AGGTAGTTCC 300  
 CAATCGCTCT AAGGAAGATC GTCGCTATTAG GACTCCACCA GTGAGATCAT TCCAAGAGA 360  
 TAATCAAAGG CAACAAAATG CTCCAAGAAA GTAAATGTTC ATATTTTAAA TAAAAAACG 420  
 10 ACAACTTAAA AAAAAAAAAA AAAAAAAAAA AAAAAAGCGG CCGCAGAATT C 471

## (2) INFORMATION FOR SEQ ID NO:2:

15 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 115 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 20  
 (ii) MOLECULE TYPE: protein  
 25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:  
 Met Thr Cys Lys Arg Arg Asn Gly Gly Arg Ala Lys His Gly Arg Gly  
 1 5 10 15  
 30 His Val Thr Pro Val Arg Cys Thr Asn Cys Ala Arg Cys Val Pro Lys  
 20 25 30  
 Asp Lys Ala Ile Lys Lys Phe Val Ile Arg Asn Ile Gly Glu Gly Ala  
 35 35 40 45  
 35 Ala Val Arg Asp Ile Thr Glu Ala Ser Val Tyr Ser Ala Tyr Val Leu  
 50 55 60  
 40 Pro Lys Leu Tyr Ala Lys Leu His Tyr Cys Val Ser Cys Ala Ile His  
 65 70 75 80  
 Ser Lys Val Val Arg Asn Arg Ser Lys Glu Asp Arg Arg Ile Arg Thr  
 85 90 95  
 45 Pro Pro Val Arg Ser Phe Pro Arg Asp Asn Gln Arg Gln Gln Asn Ala  
 100 105 110  
 Pro Arg Lys  
 115

## (2) INFORMATION FOR SEQ ID NO:3:

55 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 870 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear  
 60 (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

5 GAATTCTGCG GCCGCAATC AAGTAACACA CATCACATCA GATCACATCA CGTTAAATG 60  
 GTCCCAAAGT TCGCTCTGGT CTGCTTGGTA GCCTGCGCCT TTGTCCAGGT TGCCCTCTGC 120  
 10 GATGCTCCCA CTGACTCCAT GCAACTGCCT TCTTTACAAG AAGTTAAGGA TAAAGCCGAT 180  
 GAGGCATTGA ACAATGTCCA AACTGAATTC CACAAATTGG TTGGTGTTC AAACAACGAA 240  
 GAATTAGTCA AGAAAATCAG TGATGGAAC TACCAACTCA AGGACACTCT TTCAAACCTC 300  
 15 TTGGGAAAGC TCAACACTGA AGTTAACGAG CACGGAAAAG GATTCAAAAG ACCTGTTGGC 360  
 TTCAGTAACC ACCACTTTCA ACGAAAAAAG TCCGCGAACT TGAGCAACAA CATCCTGATG 420  
 TGAAGAAACA AGCCGATGAG GTAAAAGCTA AATTCCAAGA AACTCTGCAG ACCGTTGGTA 480  
 20 ATGAAGCGGG GAAAATTGAC TAAAGACTCG GAAAAAGTGC TTGAAGGCAT GAACGAGCAA 540  
 TTGAGCAATT TAACTAAAGA AGTTTTGACC CAAGCCCCAA CAGTGCCCCC CGACGTGCGT 600  
 25 TCCAGTGTG CCAATTCCC CAAAGAACAC GAAGCCACCC ACACCGGCCA CTAATTATAA 660  
 ACAGAAAACC TTCTGTGATC ATCATTATC ATATCCATA TATATATAA TTCATTAATT 720  
 AATTAAAATT TGTTTTATAG TTGTTTAAAT AATCTTCGGA TATTTGTAAA AATTATTTGG 780  
 30 ATGCTCATTT GATTATATT ATAAATAAAA TAATTCTATA ATATAAAAAA AAAAAAAAAA 840  
 AAAAAAAAAA GAAAAGCGGC CGCAGAATTC 870

## 35 (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

40 (A) LENGTH: 121 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

45

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

50 Met Val Pro Lys Phe Ala Leu Val Cys Leu Val Ala Cys Ala Phe Val  
 1 5 10 15  
 Gln Val Ala Leu Cys Asp Ala Pro Thr Asp Ser Met Gln Leu Pro Ser  
 20 25 30  
 55 Leu Gln Glu Val Lys Asp Lys Ala Asp Glu Ala Leu Asn Asn Val Gln  
 35 40 45  
 Thr Glu Phe His Lys Leu Val Gly Val Gln Asn Asn Glu Glu Leu Val  
 50 55 60

60

Lys Lys Ile Ser Asp Gly Thr Asp Gln Leu Lys Asp Thr Leu Ser Asn  
 65 70 75 80  
 Phe Leu Gly Lys Leu Asn Thr Glu Val Asn Glu His Gly Lys Gly Phe  
 5 85 90 95  
 Lys Arg Pro Val Gly Phe Ser Asn His His Phe Gln Arg Lys Lys Ser  
 100 105 110  
 Ala Asn Leu Ser Asn Asn Ile Leu Met  
 115 120

## (2) INFORMATION FOR SEQ ID NO:5:

- 15 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 619 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 20 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GAATTCTGCG GCCGCGATCT GGTTCAACAAG GAAGTTGTGG CTAAAGTTGA GACTGCTGTT 60  
 GCTCATGATA CTGCTGAAGT ATGGGCGGGA GATGAGCCAG CCACTCAATT ATGGACTGAT 120  
 30 GATGCACCTG TTGCTGCAGT TCCGGCTGTT TACCCAGCTG CTGCCAGCCA AGATTGGGCC 180  
 GAGCAAGTGC AAGAAGAATG GGCTGCCAAC CTACCCAGC TGCTGGTCAA ACTACTTGGG 240  
 35 GAAGCTCCAC ACAAGAATGG TCATAAATCA AATGATACTT TGGGTATTGG ATAAATGAAA 300  
 TAAATAAAAA TAAAAATAAA AAAAAAAAAA AAAACCGCCG AACTGACGG GCTCCAGGAG 360  
 TCGTCGCCGC CAATCCCCAT ATGGAAACCG TCGATATTCA GCCATGTGCC TTCTTCCGCG 420  
 40 TGCAGCAGAT GGCGATGGCT GGTTTCCATC AGTTGCTGTT GACTGTAGCG GCTGATGTTG 480  
 AACTGGAAGT CGCCGCGCCA CTGGTGTGGG CCATAATTCA ATTCGCGCGT CCCGCGAGCGC 540  
 45 AGACCGTTTT CGCTCGGGAA GACGTACGGG GTATACATGT CTAGGGGTAT CCACTATTCT 600  
 AGATGCGGCC GCTGAATTC 619

## (2) INFORMATION FOR SEQ ID NO:6:

- 50 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 76 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 55 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

60 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Ile Leu Leu Lys Tyr Gly Arg Glu Met Ser Gln Pro Leu Asn Tyr  
1 5 10 15  
5 Gly Leu Met Met His Leu Leu Leu Gln Phe Arg Leu Phe Thr Gln Leu  
20 25 30  
Leu Pro Ala Lys Ile Gly Pro Ser Lys Cys Lys Lys Asn Gly Leu Pro  
35 40 45  
10 Thr Leu Pro Gln Leu Leu Val Lys Leu Leu Gly Glu Ala Pro His Lys  
50 55 60  
Asn Gly His Lys Ser Asn Asp Thr Leu Gly Ile Gly  
15 65 70 75

WHAT IS CLAIMED IS:

1. A purified DNA composition encoding an allergen from a *Ctenocephalides* species or a fragment thereof of at least about 18 nt, as part of other than a naturally occurring chromosome.  
5
2. A purified DNA composition according to Claim 1, wherein said DNA is cDNA.
3. A purified DNA composition according to Claim 1, wherein said DNA  
10 composition comprises a transcriptional initiation region 5' of said DNA sequence.
4. A purified DNA composition according to Claim 1 wherein said allergen has an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:6.  
15
5. A purified DNA composition according to Claim 1, wherein said nucleotide sequence is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3 and SEQ ID NO:5.
8. A cell comprising a DNA composition having a sequence encoding an  
20 allergen from a *Ctenocephalides* species at other than its natural chromosomal site, or a fragment thereof of at least about 18 nt.
9. A cell according to Claim 8, wherein said allergen encoding DNA  
25 sequence is under the transcriptional regulatory control of an exogenous transcriptional initiation region.
10. A purified polypeptide composition comprising at least 50 weight % of the protein present as a Cten f polypeptide or a fragment thereof comprising a functional  
30 epitope.
11. A method of screening patients for flea specific allergies, said method comprising:



contacting a patient or patient sample with at least one Cten f polypeptide or a fragment thereof comprising a functional epitope;  
determining the presence of an IgE specific response to said polypeptide;  
wherein the presence of a response is indicative of a flea specific allergy.

5

12. A method according to Claim 11, wherein said determining of an IgE specific response comprises:

injecting said patient intradermally with a formulation comprising said polypeptide composition, and a control formulation at a different site;

10 measuring the wheal which forms at the sites of said injections;

wherein an increase in the size of said wheal at the site of injection of the polypeptide formulation as compared to the site of injection of said control formulation is indicative of a flea specific allergy.

15

13. A method according to Claim 11, wherein said determining of an IgE specific response comprises:

binding said polypeptide composition to an insoluble support;

combining a patient sample with said insoluble support;

detecting the presence of patient IgE bound to said support;

20 wherein the presence of patient IgE is indicative of a flea specific allergy.

14. A method according to Claim 11, wherein said determining of an IgE specific response comprises:

25 combining a patient sample with a filter comprising said polypeptide composition;

detecting the presence of patient IgE bound to said filter;

wherein the presence of patient IgE is indicative of a flea specific allergy.

15. A method for determining the allergic responsiveness of a host to flea allergens, said method comprising:

30 combining in an assay medium a blood sample suspected of containing IgE specific for flea allergens with a purified protein composition comprising at least one of Cten f I, II or !I, or a fragment thereof comprising a functional epitope;

incubating for sufficient time for complex formation to occur between said IgE  
35 and said protein composition; and

detecting the presence of said complex as indicative of flea allergen allergic responsiveness.

16. A method of hyposensitizing a patient having flea specific allergies, the method comprising:

5 injecting said patient periodically, at least about every 21 days, with a formulation comprising at least one Cten f polypeptide for a total of at least about 5 injections;

wherein said patient shows a decrease in allergic symptoms.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/13658

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 39/35; C12N 1/21, 15/70, 15/79, 15/86; G01N 33/53

US CL : 424/275.1, 276.1; 435/7.1, 69.3, 252.3, 254.11, 320.1; 536/23.5

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/275.1, 276.1; 435/7.1, 69.3, 252.3, 254.11, 320.1; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, CA, CABA, MEDLINE, EMBASE

search terms: Ctenocephalides, Ctenf 1

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	US, A, 5,418,137 (YAMANAKA ET AL) 23 MAY 1995, see entire document.	1, 2, 4, 5, 10
Y	Veterinary Immunology And Immunopathology, Volume 37, issued 1993, GREENE ET AL., "Isolation and In vitro translation of messenger RNA encoding allergens of the cat flea, Ctenocephalides felis", pages 15-23, see entire document.	1-16
Y	WO, A, 92/03156 (PARAVAX, INC) 05 March 1992, see entire document.	1-16
Y	WO, A, 93/18788 (OPDEBEECK ET AL) 30 September 1993, see entire document.	1-16



Further documents are listed in the continuation of Box C.



See patent family annex.

## \* Special categories of cited documents:

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\*E\* earlier document published on or after the international filing date

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\*O\* document referring to an oral disclosure, use, exhibition or other means

\*P\* document published prior to the international filing date but later than the priority date claimed

\*T\*

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\*

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

\*A\*

document member of the same patent family

Date of the actual completion of the international search

09 JANUARY 1996

Date of mailing of the international search report

15 FEB 1996

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